MICROBIAL POLYSACCHARIDES AS PLASMA SUBSTITUTES

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Introduction and Summary of Previous Work (1952)

Selection of bacteria which are potential polysaccharide producers may involve selection of strains either from groups of known capabilities or of unidentified organisms at random from their natural environment. The latter method was employed in this work, and soil from various localities was used as source material for bacterial cultures.

Primary isolations, using various physical and nutritive conditions for growth, yielded about 750 cultures of bacteria. Approximately 175 of these were used to determine conditions for polysaccharide production for screening purposes. The effects of time and temperature of incubation, aeration, sugar and nitrogen concentration, and final pH of the medium on production of polysaccharides were tested. From the results of these experiments (see Report, 1952), conditions were selected for the primary screening. The remainder of the culture collection was then passed through the screening program. Because the yeast-tryptone medium used hindered osmotic pressure determinations and recovery of polysaccharide for the biochemical work, an inorganic salts medium and a semi-synthetic medium were tried; these media are cited in the Report 1952. It will be noted that the salts medium is restricted in nutrients whereas the semi-synthetic medium provides a complex of growth factors, such as required by fastidious microorganisms like the lactobacilli. Experiments were done in which primary isolations from soil were made on the salts and semi-synthetic agars, with subsequent transfer of the cultures into homologous broths for polysaccharide production. Such a procedure might be expected to give cultures better adapted to the media and, therefore perhaps, capable of producing more polysaccharide.

Methods

A. Original isolation. One gram of soil was placed in 99 ml of sterile water and vigorously shaken. The suspension thus well-separated was diluted to the extent required to produce colonies upon plating, and then transferred aseptically to sterile Petri dishes in triplicate for each dilution to be used. About 15 ml of the melted agar chosen for plating, cooled to 45°C, was then added and the inoculum mixed by swirling. All plates were incubated, usually aerobically at 25, 30 or 37°C until growth occurred. Transfers were then made onto agar slants from isolated colonies taken at random, incubated and, after check of purity, were stored until used.

B. Tests for polysaccharide production. Test tubes of 25 X 200 mm, containing 10 ml of the medium to be tested, were inoculated by loop from 18 to 24 hr old agar slants of the proper organisms. The tubes were placed on a rotary shaker for 2-3 days at 30°C, after which they were centrifuged to remove suspended matter and cells. Then 0.5 ml of the supernatant was dialyzed against cold running water for 12-18 hrs. The non-dialyzable material was then diluted and tested with anthrone reagent for polysaccharide, and the results expressed as mg polysaccharide per ml of original culture. More recently, analyses for polysaccharides have been done by the methanol method developed in the Biochemistry Department. Because of the saving of time with this latter method, it is now preferred.

Test of Cultures Isolated on Salts and Semi-synthetic Media

Approximately 250 oultures were isolated on salts and semi-synthetic agars and then were tested in homologous broths for polysaccharide production. The results of this work, only partly finished in the previous annual report, are now complete and summarized in Table 1.

Table 1

Polysaccharide production by cultures isolated and grown on salts and semi-synthetic media

Polysaccharide range mg/ml	Salts			Semi-synthetic		
	No. of cultures	Per cent of total		No. of cultures	Per cent of total	
0-5	152	61		180	72	
5-10	50	20	39	42	17	28
10+	49	19		28	11	

In the previous report (1952), at the bottom of page 5, preliminary results are listed which have bearing upon those in Table 1. That is, the values recorded in both tables were obtained from cultures which were transferred from the agar used for isolation into the corresponding broth for polysaccharide production. Comparison of results in the two tables under the heading of "Per cent of total" shows that only 15% of the cultures from the yeast-tryptone agar in the early work produced better than 5 mg/ml of polysaccharide, whereas with the recent procedure with the salts and semi-synthetic media, 39% and 28% respectively produced comparable amounts. These results may indicate that the 250 recently isolated were better adapted to the salts and semi-synthetic broths, since about twice as many of them produced reasonably large amounts of polysaccharides in these media as did the random cultures isolated on the yeast-tryptone medium. Similarly the yield of cultures producing 10 + mg/ml was 2 to 3 times greater with the new procedure.

Some of the above-mentioned 250 cultures were isolated from soil on salts agar and some on semi-synthetic agar. The two isolation media might well result in different types of cultures in the collections concerned, and so it was necessary to test their polysaccharide production as shown in Table 2 for those isolated on salts agar and in Table 3 for those isolated on the semi-synthetic agar.

Apparently the type of medium which was used for the original isolation was not a critical factor, as long as the organisms were grown in salts broth for polysaccharide production. Cultures isolated on the semi-synthetic agar did not seem to require more complex factors for growth than did those isolated on the salts agar. As will be shown later, the two collections were proved to contain mainly bacilli and were surprizingly restricted in species.

Table 2

Polysaccharide production by organisms isolated on salts medium

Polysaccharide range mg/ml	Medium of test			
	Salts		Semi-synthetic	
	No. of cultures	Per cent of total	No. of cultures	Per cent of total
0 - 5	49	57	51	60
5 - 10	19	22	26	31
10+	18	21	8	9

Table 3

Polysaccharide production by organisms isolated on semi-synthetic medium

Polysaccharide range mg/ml	Medium of test			
	Salts		Semi-synthetic	
	No. of cultures	Per cent of total	No. of cultures	Per cent of total
0 - 5	103	62	125	76
5 - 10	33	20	17	10
10+	31	18	23	14

Reproducibility of Results

Experiments testing the reproducibility of the results obtained have been performed with 99 cultures isolated under the above conditions, i.e., on salts and semi-synthetic media. Approximately two months after the cultures were originally tested for production of polysaccharides on the homologous salts or semi-synthetic broths, they were retested under the same conditions. The results are shown in Tables 4 and 5. From Table 4 it is seen that the results in both series of tests were very similar, if the 99 cultures are considered as a whole. However, Table 4 does not show whether the same cultures fell into the same category in the re-test. The answer to the question is found in Table 5 which shows how the individual cultures varied. About three-fourths of the cultures studied produced the same range of polysaccharide in the two experiments, although there was slightly more variation in the cultures in semi-synthetic for reasons which are not known. It must be considered when reviewing Table 5 that the figures in the three columns may be greatly affected by a change of only 1 mg/ml of polysaccharide if that change occurs at the points of separation of the three ranges, whereas a larger variation remote from one of the points of separation would not be recorded as a change from one range to another. Thus the significance of the few data presented may be questioned but our impression from experience is that a high producing culture is reasonably stable.

Table 4

Reproducibility of grouping of cultures into three ranges on polysaccharide production

Grown on	Per cent of cultures producing polysaccharide in mg/ml 0 - 5	5 - 10	10 +
Salts medium			
Exp. A	58	24	18
Exp. B	59	24	17
Semi-synthetic medium			
Exp. A	59	26	15
Exp. B	53	25	22

Table 5

Reproducibility in range of polysaccharide produced by individual cultures

Grown on	Per cent of cultures producing polysaccharide in Lower range	Same range	Higher range
Salts medium	11	78	11
Semi-synthetic medium	9	67	24

Identification of Cultures

On the basis of their polysaccharide production 140 of the isolates were selected for taxonomic study. Table 6 summarizes the characteristics used to classify these isolates. By the classifications of Bergey (6th Edition) and of Smith, Gordon and Clark (1946), the cultures fall into 11 groups whose species identity is given in the table.

Although the cultural appearance of the B. subtilis group in the collection studied was not that commonly associated with B. subtilis, the members of this group were similar inter se. The selection for polysaccharide synthesizing strains may have resulted in choosing within a restricted group. Upon cultural characteristics as a basis of division, the isolates fall into two groups. One type forms round to irregular, raised, smooth glistening colonies with a hard core. The colony has a runny consistency and may or may not spread. The other type forms an irregular, smooth, dull, flat, spreading colony with concentric rings caused by variation in opacity of the colony. These differences suggest smooth/rough variation, which should be kept in mind in stock culture maintenance, as it may affect polysaccharide production.

Table 6

Reaction of isolates to tests employed for taxonomic purposes

	No. of isolates	Morphology and Grem reaction	Spores	Catalase	AMC produced	Growth at pH 6	Gelatin hydrolysis	Xylose utilized	Arabinose utilized	Glucose utilized
Group I	36	rods, 1 u in diam., Gram +	+	+	+	+	+	A	A	A
Group II	17	rods, 1 u in diam., Gram +	+	+	+	+	+	A	-	A
Group III	4	rods, 1 u in diam., Gram +	+	+	+	+	+	?	?	A
Group IV	11	rods, 1 u in diam., Gram +	+	+	+	+	-	A	A	A
Group V	27	rods, 1 u in diam., Gram +	+	+	+	+	+	A	A	A
Group VI	4	rods, 1 u in diam., Gram +	+	+	+	+	+	A	-	A
Group VII	4	rods, 1 u in diam., Gram +	+	+	+	+	+	A	A	A
Group VIII	3	rods, 1 u in diam., Gram +	+	+	+	+	+	A	A	A
Group IX	9	rods, 1 u in diam., Gram +	+	+	-	+	+	A	A	A
Group X	1	rods, 1 u in diam., Gram ±	+	+	+	+	+	A + G	A + G	A + G
Group XI	8	rods, 1 u in diam., Gram +	+	+	+	+	+	-	-	A

Table 6 continued

	Starch hydrolyzed	Nitrate reduction	Citrate utilization	Growth on glucose-NO_3 medium	Pigment on tyrosine agar	Anaerobic glucose dissimilation	Species identity
Group I	+	+	+	darkening of medium	–	–	B. subtilis var. aterrimus
Group II	+	+	+	darkening of medium	–	–	" " " " biotype
Group III	+	+	+	darkening of medium	–	–	" " " " biotype
Group IV	+	+	+	darkening of medium	–	–	" " " " biotype
Group V	+	+	+	+	–	–	B. subtilis
Group VI	+	+	+	+	–	–	" " biotype
Group VII	+	–	+	+	0	0	B. subtilis-pumilus intermediate
Group VIII	–	–	+	+	0	0	B. pumilus
Group IX	+	±	+	mucoid +	0	0	B. megaterium
Group X	+	+	–	–	0	0	B. polymyxa
Group XI	+	+	+	–	0	0	B. cereus

0 = no data

The majority of the cultures which are capable of producing greater than 5 mg/ml of polysaccharide are in the B. subtilis or B. subtilis var. aterrimus species (Table 7).

Table 7

Amount of polysaccharide produced by identified isolates

Polysaccharide mg/ml	B. subtilis var. aterrimus	B. subtilis	B. subtilis-pumulis intermediates	B. pumilus	B. megaterium	B. cereus	B. polymyxa
20+ above	1*	1	0	0	0	0	0
15 - 19	12	1	1	0	0	1	0
10 - 14	21	12	0	0	2	1	0
5 - 9	28	15	3	0	3	2	1
5	1	0	0	3	4	3	0

* number of isolates.

Other investigators (Forsyth and Webly, 1949 and Owen, 1923) have reported that most members of the genus Bacillus produce polysaccharide. It is interesting that the isolating procedure used here resulted in such a homogeneous collection of the B. subtilis group rather than random bacilli or other soil organisms.

The biochemical tests further selected within the cultures and resulted in choice of seven cultures of greatest interest. Six of the seven were found to be members of the genus Bacillus. These are cited by strain numbers and species or variety names:

No.	
1904 2705 248	B. subtilis var. aterrimus
1624 A5	B. subtilis-pumilus intermediates
1605	B. cereus

The remaining culture which had passed the biochemical screening was a Pseudomonas sp., unidentified because no further work was done with it.

Effect of Nitrogen Level on Polysaccharide Production

Because of the possibility of limiting growth by low concentrations of nitrogen in the medium, thus resulting in small cell population and perhaps less polysaccharide, the effect of nitrogen level has been examined. In the preliminary work, 150 ml of the salts broth was placed in Erlenmeyer flasks, one

lot containing 350 mg of N per liter and the other only 35 mg of N per liter. The nitrogen source was Difco vitamin-free casamino acids. Duplicate flasks were inoculated with one of the seven above organisms and placed on a rotary shaker at 30°C. At 12, 25, 37 and 48 hours growth the cultures were examined for polysaccharide and turbidity as an index of growth. Results are shown in Tables 8 and 9. The effect of high total nitrogen levels is evidenced in both tables by increased amounts of polysaccharide (Table 8) and by greater optical densities (Table 9) of the cultures. These results show in addition that a direct correlation cannot be made between turbidity and the amount of polysaccharide produced by a culture.

Table 8

Polysaccharide production in media of different nitrogen content

Age,	A5		1904		1605		2705		1624		1719		248	
hours	A	B	A	B	A	B	A	B	A	B	A	B	A	B
12	8	7	*	*	-	-	-	-	-	-	*	-	-	-
25	4	4	-	-	-	8	-	2	8	9	-	-	-	-
37	6	9	4	5	-	10	5	7	11	13	3	5	4	5
48	7	13**	4	8	4	17	7	15	11	15	4	6	4	9

A = salts broth containing 35 mg N/liter
B = " " " 350 mg N/liter
* = not analyzed
- = too low for analytical value by the anthrone method
** = in other experiments the range of 10-11 mg/ml was commonly found.

Table 9

Turbidity* of cultures in media of different nitrogen concentration

Age,														
hours	A	B	A	B	A	B	A	B	A	B	A	B	A	B
12	.545	.752	.090	.068	.240	.456	.038	.021	.096	.246	.056	.171	.050	.053
25	.678	.783	.301	.444	.347	.979	.143	.332	.699	.846	.301	1.071	.352	.581
37	.721	.699	.426	.462	.441	1.188	.620	.770	.796	1.071	.450	1.350	.450	.743
48	.710	.673	.475	.505	.444	1.262	.633	.715	.796	1.262	.438	1.374	.492	.710

A = salts broth containing 35 mg N/liter
B = " " " 350 mg N/liter
* = read in Evelyn photometer and recorded as optical density.

Polysaccharide Synthesis by Growing Cultures of Strain A5

A5, a B. subtilis-pumilus intermediate, was studied further to determine the optimum conditions for polysaccharide production by this strain. Because the original salts medium was apparently limiting growth and possibly polysaccharide synthesis, the effect of raising the nitrogen concentration was investigated.

A series of 500 ml Erlenmeyer flasks containing 200 ml of the salts medium with concentrations 20, 100 and 1000 ug/ml of nitrogen in the form of $(NH_4)_2SO_4$ or vitamin-free casamino acids were inoculated with a 3 per cent by volume dense cell suspension of culture A5 and incubated on a rotary shaker at 30°C. Ten ml samples were taken at 0, 12, 16, 24, 36 and 48 hrs., the cells killed with flowing steam and removed from the culture broth by centrifugation. The cell nitrogen content was determined by a Kjeldahl method (Kabat et al., 1948). The supernatant culture broth was analyzed for polysaccharide, reducing sugar, protein, and total carbohydrate content. The polysaccharide analysis was done by the methanol method developed by the Biochemistry Dept., the reducing sugar by the method of Nelson (1944), and the protein by a colorimetric method using the Folin-Crocatheau phenol reagent (Kabat et al., 1948). The residual sucrose was determined by subtracting the values obtained in the polysaccharide and reducing sugar analyses from the total carbohydrate determined by the anthrone method on an aliquot of the culture broth. Because of the method of determination, the residual sucrose values are undoubtedly inaccurate but they serve to indicate the general trend of sucrose disappearance. The pH of the cultures was determined with a Beckman pH meter.

It can be seen in Figures 1-6 that the level of polysaccharide produced by all cultures did not vary greatly. The time required to synthesize this amount of polysaccharide was less in media with high levels of nitrogen. This is probably caused by the larger population present to synthesize the polysaccharide rapidly. However, when the rates of polysaccharide formation by the different cultures are compared, it can be seen that the rate of polysaccharide synthesis is much higher if the nitrogen concentration of the medium is low. The lower rate of polysaccharide synthesis in a culture grown in a high nitrogen medium may be caused by the change in pH which occurs in the high nitrogen culture. The sucrose, however, disappears much more rapidly in the cultures with a high nitrogen concentration and consequently a high population. The greater utilization of sucrose is probably caused by the greater assimilatory demand of the larger population. The lack of substrate available to the levansucrase enzyme system, caused by the use of the substrate for assimilatory purposes, could lower the rate of formation of polysaccharide. Another possible explanation of the lower rate of polysaccharide formation by cells growing in a high nitrogen medium is that the actual rate of synthesis is the same, but that the large population hydrolyzes some of the levan for use as assimilatory carbohydrate, after the amount of sucrose available for this purpose is diminished. If the nitrogen concentration of the medium is high, hydrolysis of the polysaccharide does occur after 16 hrs. growth of the culture (see Fig. 5 and 6), at which time the sucrose is almost gone.

A change of kind of nitrogen substrate at the same concentration did not appear to affect the amount of polysaccharide formed, the time required to obtain maximum yield, or the rate of formation.

Acoording to Hestrin et al. (1944), the reducing sugar found in culture broth of A. levanicum is the glucose part of the sucrose molecule, formed as a result of the levansucrase splitting of the sucrose, and the glucose should accumulate as the levan is formed. Since culture A5 oxidizes glucose as readily as sucrose, the amount of glucose accumulating in the culture broths would not necessarily be proportional to the amount of levan being formed. However, the amount of reducing sugar in the culture broth does tend to parallel the amount of polysaccharide present.

Fig.1 Changes occurring with the nitrogen at 20μg per ml as $(NH_4)_2SO_4$
(culture A5, 48 hrs. growth, salts medium)

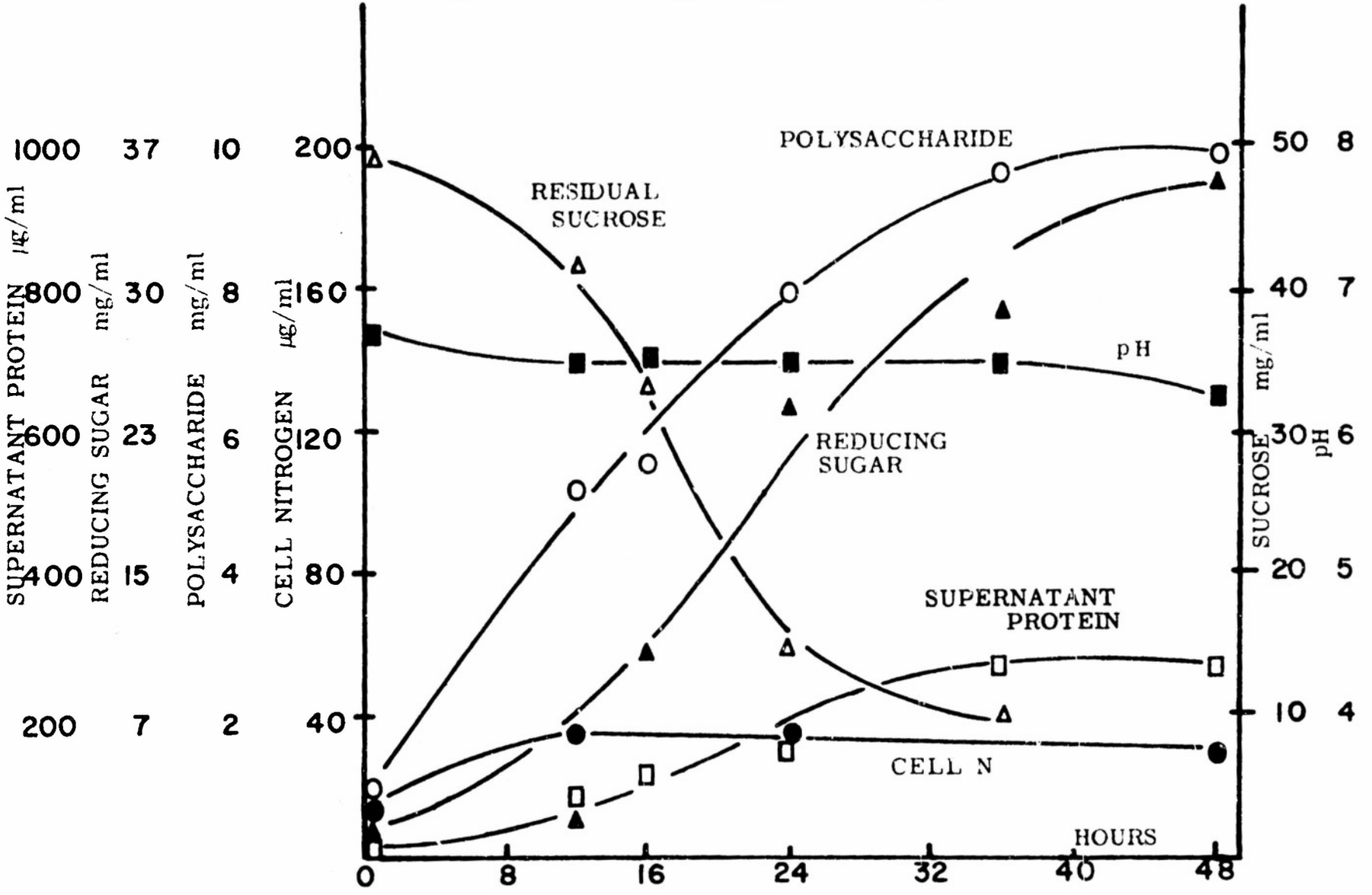


Fig. 2 Changes occurring with the nitrogen at 20μg per ml as casamino acids.
(culture A5, 48 hrs. growth, salts medium)

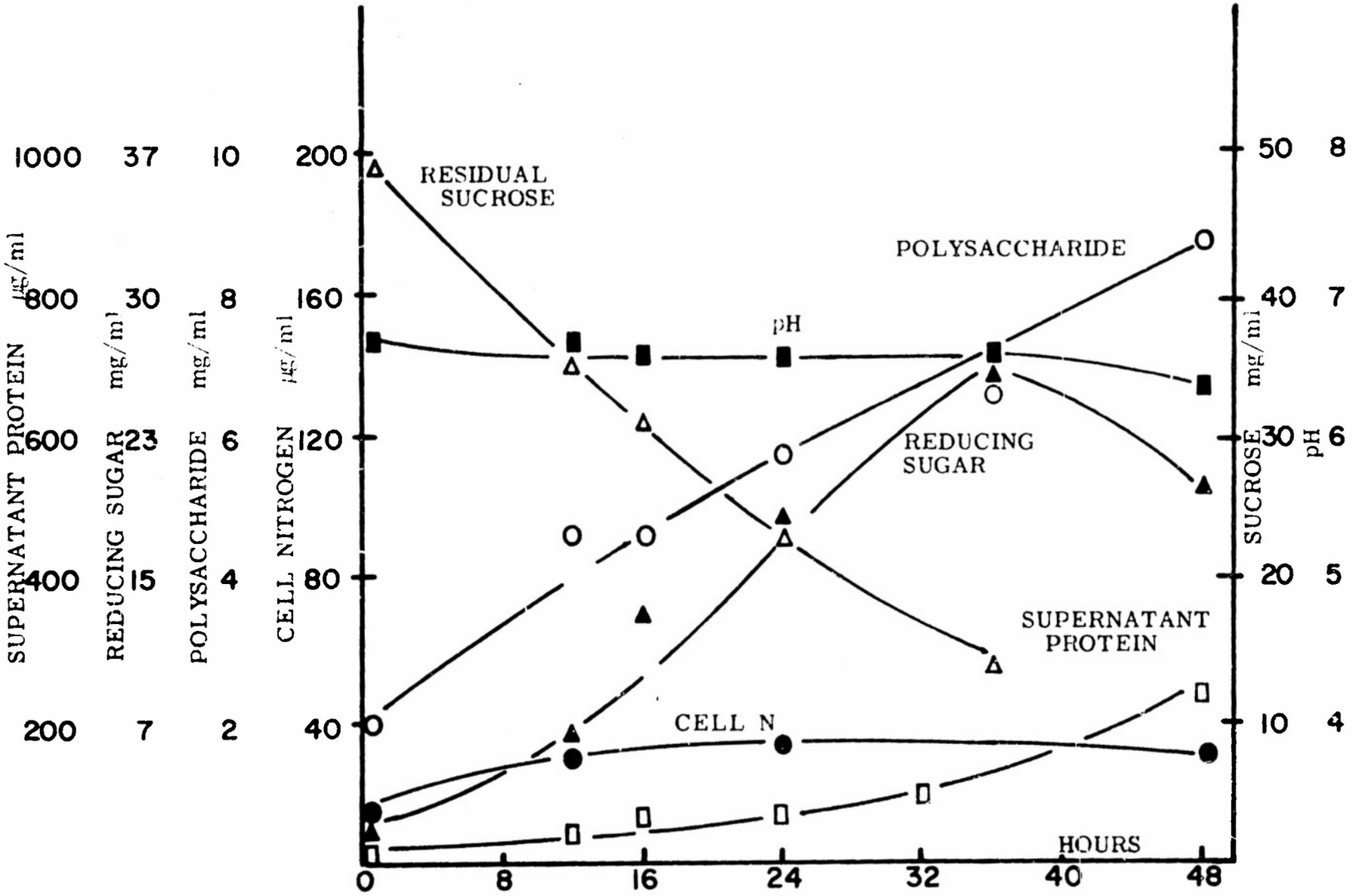


Fig. 3 Changes occurring with the nitrogen at 100 μg per ml as $(NH_4)_2SO_4$
(culture A5, 48 hrs. growth, salts medium)

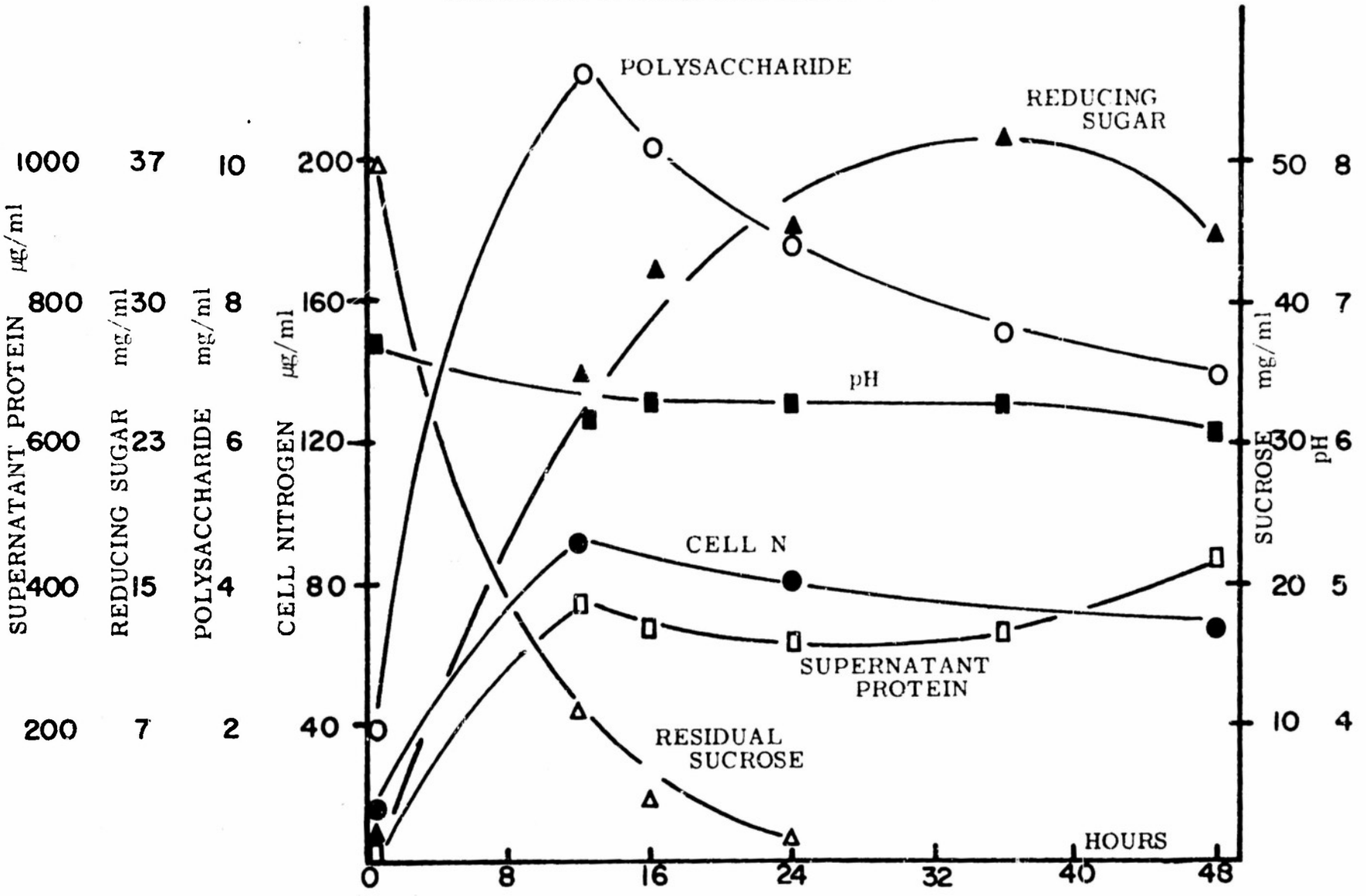


Fig.4 Changes occurring with the nitrogen at 100μg per ml as casamino acids.
(culture A5, 48 hrs. growth, salts medium)

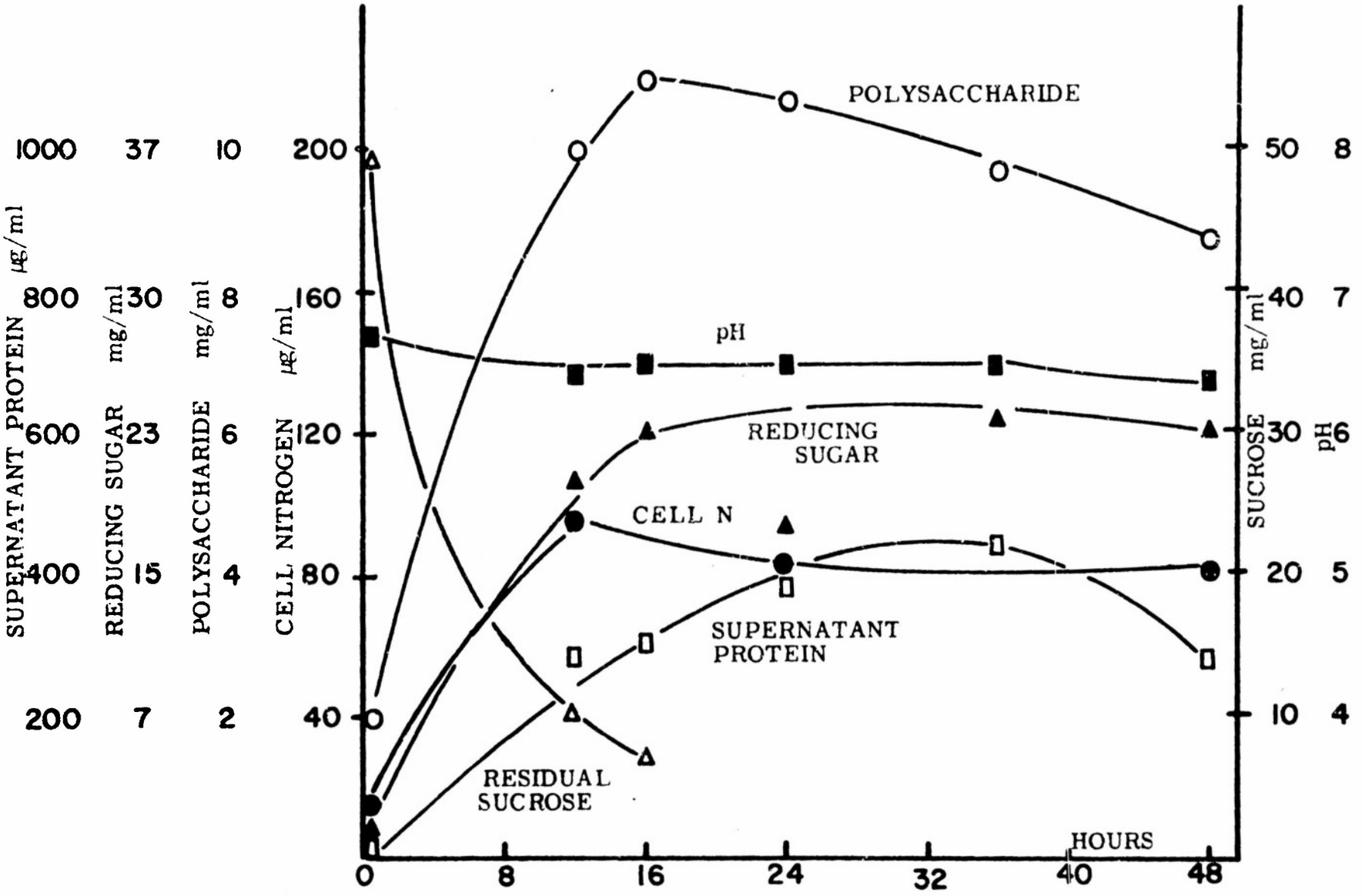


Fig. 5 Changes occurring with the nitrogen at 1000μg per ml as $(NH_4)_2SO_4$.
(culture A5, 48 hrs. growth, salts medium)

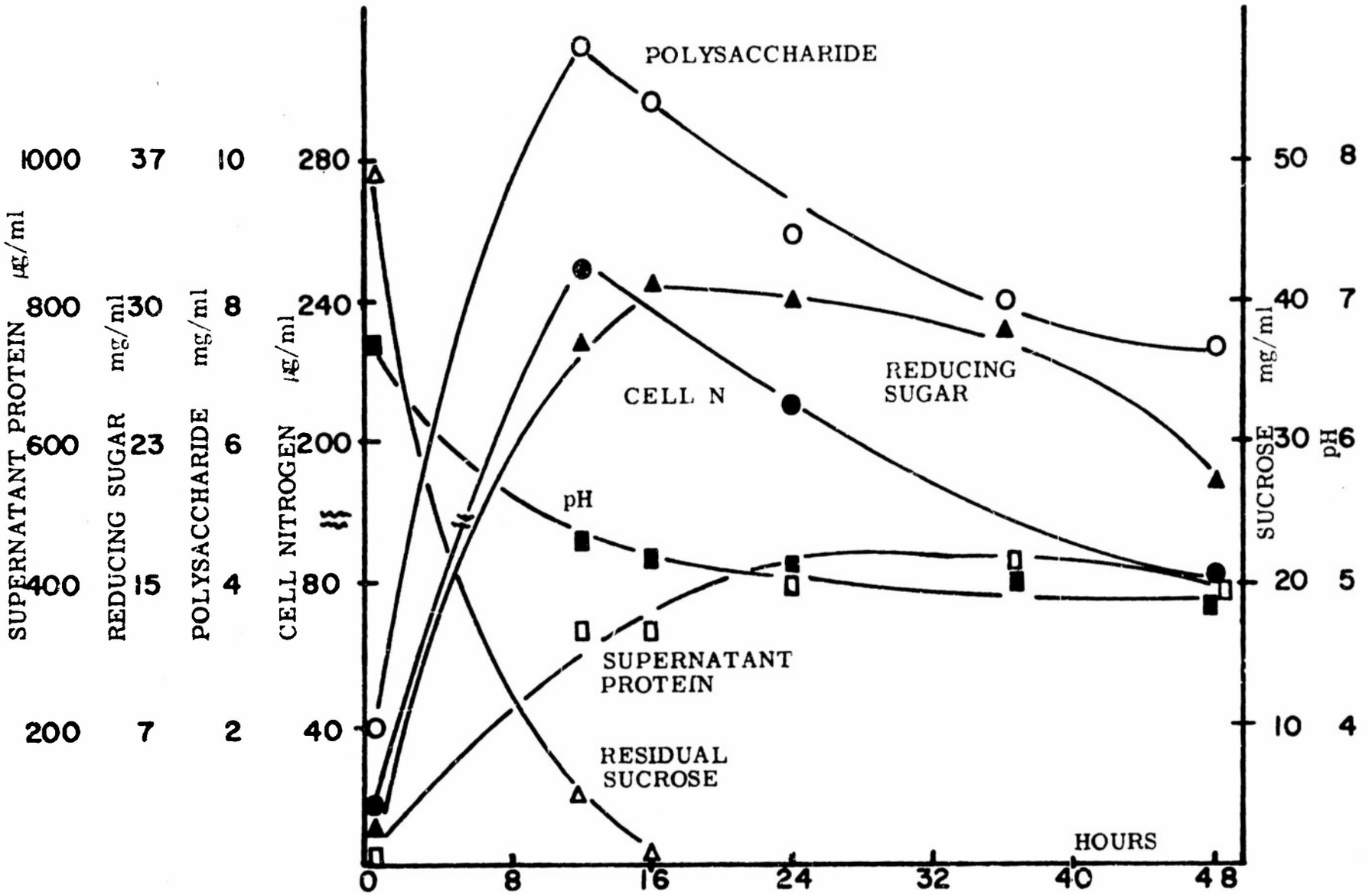
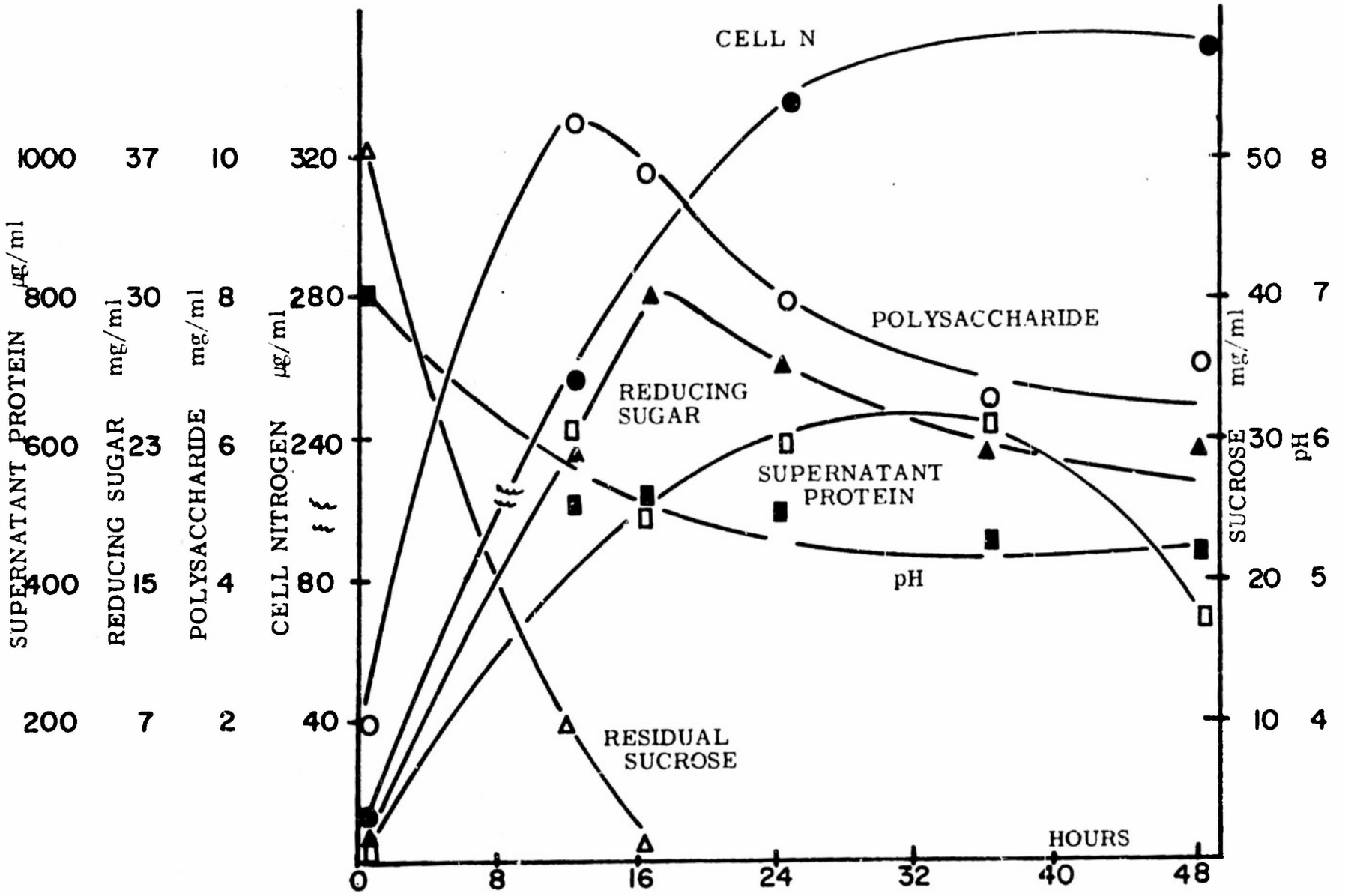


Fig. 6 Changes occurring with the nitrogen at 1000 μg per ml as casamino acids.
(culture A5, 48 hrs. growth, salts medium)



Because the levansucrase enzyme system is considered to be extracellular (Aschner et al., 1942), an analysis of protein in the supernatant culture broth was attempted. Although there may have been some interference by protein from autolysis of cells, the curve for protein in the supernatant does not appear to represent protein from autolysis.

Polysaccharide Synthesis by Resting Cells of Strain A5

Because of the ease of studying conditions for polysaccharide formation with resting cell preparations, the possibilities of using such a technique were investigated. The organisms were grown in 200 ml of the salts medium in 500 ml Erlenmeyer flasks with an inoculum of 3 per cent by volume of a 24 hr. culture in the same medium. When the culture was 20 hrs. old, the cells were removed by centrifugation, washed twice with sterile water, and resuspended in a salts solution of the same composition as the medium except that the nitrogen source and sucrose were excluded. An aliquot of the cell suspension was pipetted into a 25 by 200 mm test tube, a sucrose solution to give 5 per cent final concentration was added, and the volume made up to 12 ml with the salts solution. An endogenous control accompanied the test cell suspension and both were incubated on a rotary shaker at 30°C. Samples were taken at 0, 15, 30, 60 and 120 minutes, the cells killed with flowing steam and removed from the preparation by centrifugation. The supernatant solutions were then analyzed for polysaccharide. From the data in Table 10 it can be seen that about 10 mg/ml of polysaccharide were synthesized in a two hour period. This amount in so short a time suggests that a further study of synthesis of polysaccharide by resting cells would be of interest.

Table 10

Polysaccharide produced by resting cells of strain A-5

	Sample--time taken in minutes				
	0	15	30	60	120
	Polysaccharide formed--mg/ml*				
On sucrose substrate	2.6	4.4	6.4	10.6	12.1
Endogenous	2.0	2.0	2.5	3.1	2.6

* Av. from 2 experiments.

Summary

1. The isolation procedure with plating on the salts medium as described yields slightly higher percentage of high-producing strains than did the isolations on the semi-synthetic medium and considerably higher than on the yeast tryptone medium previously reported.

2. Isolation on either the salts or semi-synthetic agars yielded a large proportion of bacilli and particularly the Bacillus subtilis group. Taxonomic study of some 140 best-producing cultures revealed 95 of B. subtilis, B. subtilis var. aterrimus, or B. subtilis-pumilus intermediates. Most of the cultures found to synthesize more than 5 mg/ml of polysaccharide are of the B. subtilis group.

3. The yields of polysaccharide were classified as to the ranges 0-5, 5-10, and 10 + mg/ml under conditions of the test upon growing cultures in salts broth with 5 per cent sucrose. About 20 per cent of the cultures tested gave yields of 10 + mg/ml.

4. Of the cultures giving 5 mg/ml and higher, which were sent to the biochemical screening, six bacilli and one Pseudomonas sp. were chosen. One culture, A5, was particularly studied for biochemical changes during polysaccharide production in cultures growing in the salts medium.

5. Polysaccharide production was also tried with resting cell preparations in N-free salts medium with 5 per cent sucrose, the cells being aerated on the shaker at 30°C. Yields of about 10 mg/ml were obtained in 2 hours.

References

Aschner, M., Hestrin, S., and Avineri-Shapiro, S. 1942 Enzymatic synthesis of levan. Nature, London, 149, 527.

Breed, R. S., Murray, E. G. D., and Hitchner, A. P. 1948 Bergey's Manual of determinative bacteriology. 6th Ed., Williams and Wilkins, Baltimore, pp. 704-762.

Forsyth, W. G. C., and Webly, D. M. 1949 Polysaccharides synthesized by aerobic mesophilic spore-forming bacteria. Biochem. J. 44, 455-459.

Hestrin, S., and Avineri-Shapiro, S. 1944 The mechanism of polysaccharide production from sucrose. Biochem. J. 38, 2-10.

Kabat, E. A., Mayer, M. M. and Heidelberger, M. 1948 Experimental immunochemistry. Thomas, Springfield, pp. 282-283, 321-323.

Nelson, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Owen, W. L. 1923 A study of the formation of gum levan from sucrose. J. Bact. 8, 421-445.

Smith, N. R., Gordon, R. E., and Clark, F. E. 1946 Aerobic mesophilic spore forming bacteria. U.S.D.A. Misc. Publication, No. 559.

BIOCHEMISTRY SECTION

Introduction

In last year's annual report, a screening procedure was outlined for the isolation of microorganisms which produce polysaccharides that might be useful as plasma substitutes without extensive preparation. This report describes improvements in the screening procedure, and provides data obtained by the application of this procedure to cultures received from the Department of Bacteriology.

Since it is recognized that microbial synthesis of polysaccharides is dependent upon environmental conditions, it was of interest to investigate the effect produced by varying some of these conditions with one particular soil isolate. Strain 248, one of the microorganisms which passed the screening procedure, was selected for this purpose.

The polysaccharide obtained from the western larch tree, as indicated in last year's annual report, passed all the preliminary requirements for a plasma substitute. Additional studies have been made to evaluate this polysaccharide further.

Experimental

The Screening Procedure

A. Improvements in large-pore membranes. In an attempt to improve upon the large-pore membranes described in the previous report (1), additional membranes were tested. A membrane was sought, the excretion behaviour of which would approach that of the human kidney with its excretion threshold of 24,000 to 40,000 (2). It was hoped that with this membrane we might be able to approximate, in about 96 hours' dialysis, the amount of dextran excreted from the kidneys in 24 hours. The time of 96 hours was chosen because in most of the excretion vs. time graphs plotted for these membranes, a plateau was obtained in that time.

The 3 per cent crude agar membrane, which was used in the earlier screening, allowed the passage of too much material. Therefore an attempt was made to find a membrane of smaller pore diameter. Air-drying of the 3 per cent crude agar membrane for one hour at room temperature had little effect. The dialysis of R-207 through various membranes of Bacto agar and crude gelatin was next studied (Figure 1). This dextran was used because, although it was not a clinical sample, it was available in large quantity. Also, it was of about the correct size and could be used satisfactorily for comparative purposes. It was noticed from the results that, at least in this instance, membranes made with Bacto agar had larger pore diameters than comparable ones made with crude agar (see Figure 2 of the last report (1)). However, the better reproducibility of Bacto agar makes it more desirable in spite of its slightly larger pore diameter. The only membranes which showed a considerable decrease in pore diameter were 5 per cent crude gelatin plus 1.5 per cent Bacto agar, and 10 per cent crude gelatin. The tendency for membranes made of gelatin alone to leak led to further work on combinations of gelatin with agar. Five per cent crude gelatin with 1.5 per cent crude agar was tested with the English and Swedish dextrans (1) and also with several other dextrans on which molecular weight data were available (Figure 2). Dextran NRC-2, a very sharp fraction with a weight average molecular weight of 11,000, had been found in clinical tests to be excreted to the extent of about 64 per cent in 24 hours (3). N-384-1C1, in which the 24 hour excretion would probably be less than 10 per cent, had a weight average molecular weight of 180,000 (3). Lot 481, which had been satisfactory clinically, had a weight average molecular weight of about 65,000, while lot 413, which had been excreted much too rapidly, had a weight average molecular weight of about 47,000 (4). Since the amount of NRC-2 left after 96 hours' dialysis, where the plateau had been reached, was considerably less than the amount left after 24 hours' excretion, it

FIGURE 1

DIALYSIS OF DEXTRAN R207 THROUGH AGAR AND GELATIN MEMBRANES

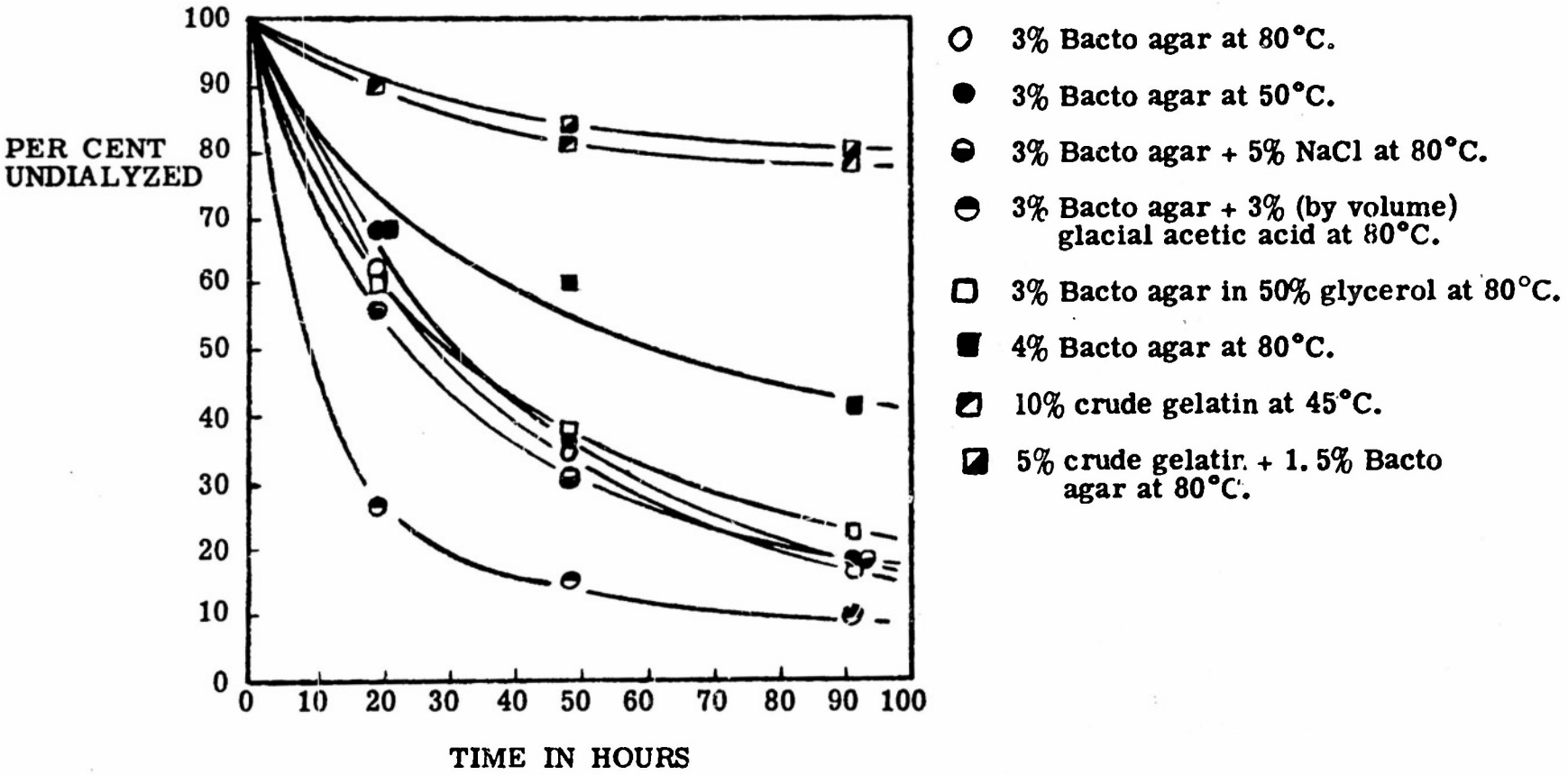
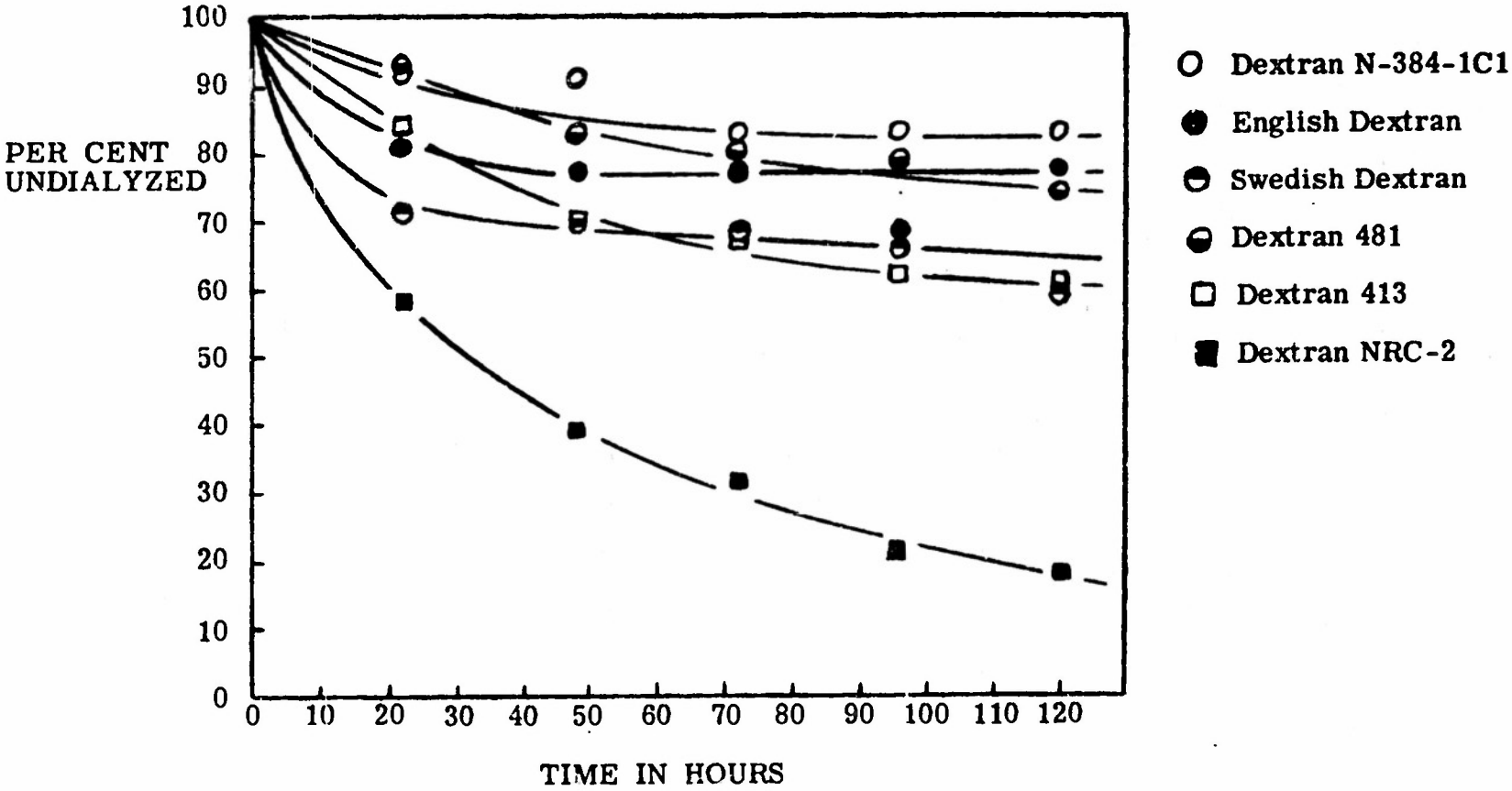


FIGURE 2

DIALYSIS OF DEXTRANS THROUGH 5% CRUDE GELATIN + 1.5% CRUDE AGAR



was apparent that this membrane was not yet of sufficiently small pore diameter.

Further combinations of gelatin and agar were tested with NRC-2, the only sample for which exact excretion data were available (Figure 3). The results show that the differences in this case between crude and purified agar and gelatin are of relatively small magnitude. Several membranes (2 per cent Bacto agar plus 5 per cent Knox gelatin, 1.5 per cent Bacto agar plus 7 per cent Knox gelatin, 2 per cent Bacto agar plus 7 per cent Knox gelatin, and 1.5 per cent Bacto agar plus 10 per cent Knox gelatin) appear to allow the passage, in 96 hours, of amounts of this sample approximately equivalent to the 24 hour human excretion figure. The six clinical dextrans were next tested with three representative membranes made with Knox gelatin and Bacto agar. For comparison, bovine plasma albumin (0.5 per cent solution in 1 per cent potassium phosphate, pH 7.4) was dialyzed through the same three membranes against 1 per cent potassium phosphate, pH 7.4, at 4° C. The phosphate buffer was changed every 24 hours. Protein analysis was by the micro-Kjeldahl method of Johnson (5). The results of 95 hours' dialysis are shown in Table I.

Table I

Dialysis of Known Samples Through Gelatin-Agar Membranes*

	Per cent Undialyzed after 95 Hours		
Sample	5% gelatin + 1.5% agar	7% gelatin + 2% agar	10% gelatin + 2% agar
Bovine plasma albumin	78	—	95
N-384-1C1	96	99	96
English (Tell No. 51C02)	73	85	92
Swedish (Macrodex No. 6793A)	65	77	85
481	78	92	88
413	70	81	93
NRC-2	20	39	62

* Knox gelatin and Bacto agar.

The membrane containing 7 per cent gelatin and 2 per cent agar most closely approximates the 24 hour clinical excretion figure for NRC-2, and if one wishes to use the membrane most closely approximating the kidney, this would be the one of choice. However, in a screening procedure it is generally advisable to retain samples which are slightly outside the immediately useful size range. On this basis, and on the basis of the albumin dialysis, which is directly related to the retention of material by the blood vessels, the membrane of choice is the one containing 10 per cent gelatin and 2 per cent agar.

B. Screening results. One thousand cultures were isolated and tested by the Department of Bacteriology. Of these cultures, 88 were found to produce polysaccharides in sufficient yield to warrant further investigation. Seven of these 88 cultures produced polysaccharides which passed the screening procedure, and are therefore considered to have possible plasma substitute properties. A brief summary of the number of cultures passing the various steps in the screening procedure is given in Table 2.

Certain physical properties of the 7 cultures that passed the screening procedure, of several clinical dextrans, and of blood plasma are given in Table 3. It is apparent that the R values of the screened polysaccharides are all in the same general range as those of the clinical dextrans.

FIGURE 3

DIALYSIS OF DEXTRAN NRC-2 THROUGH GELATIN-AGAR MEMBRANES

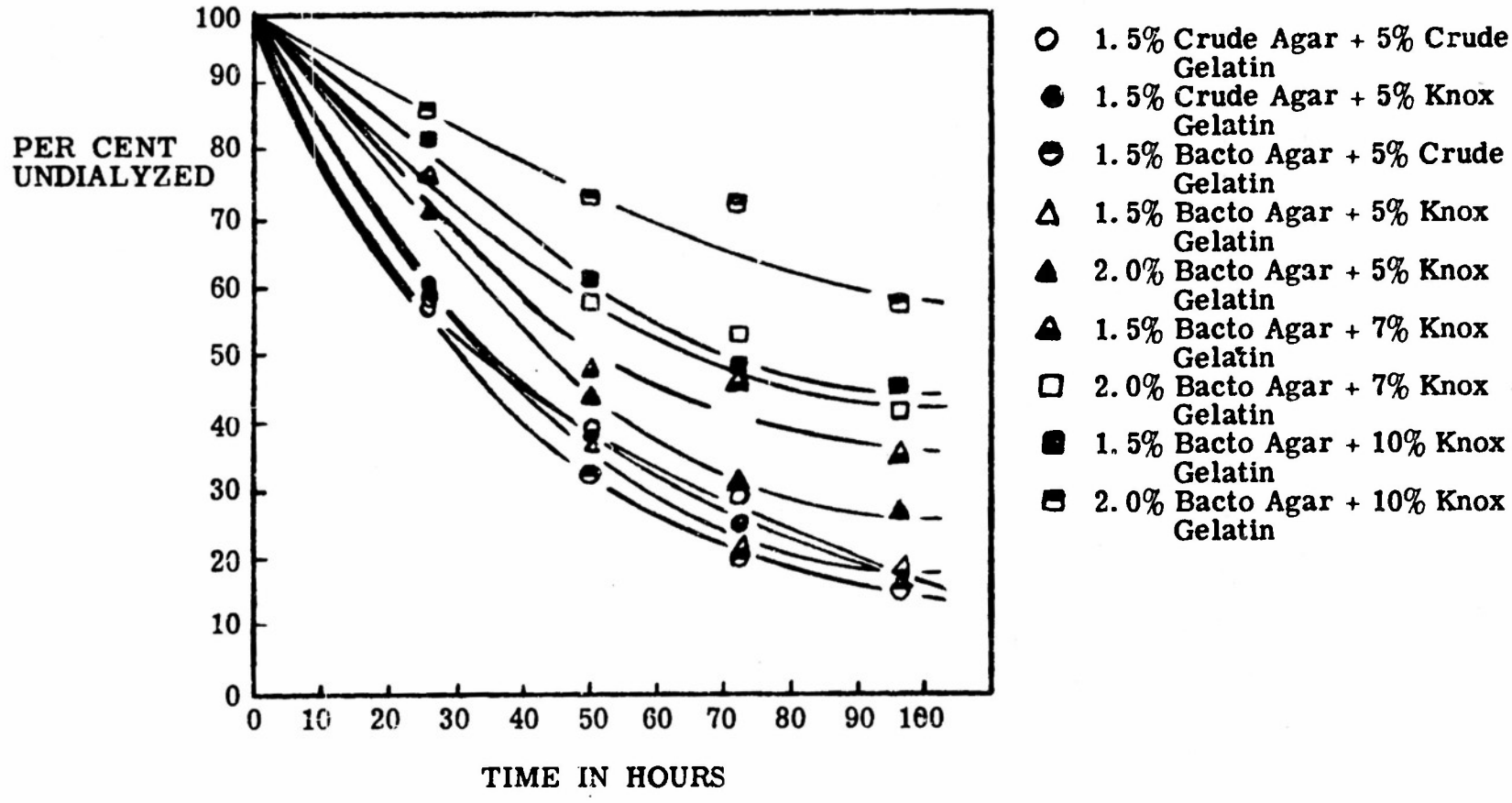


Table 2

Results of Screening Procedure

Test	No. of cultures passing test
Total no. tested	1000
Preliminary screening in Bact. Dept.	88
Large pore dialysis	55
Viscosity	44
Osmotic pressure and "R" value	7

Table 3

Physical Properties of Several Potential Plasma Substitutes

Sample	Osmotic pressure of 1% solution in mm. Skellysolve C	Intrinsic viscosity	R
American Dextran	68	0.267	256
English Dextran	48	0.290	165
Swedish Dextran	49	0.175	254
Blood Plasma	—	—	1300
Strain 248	85	0.507	168
Strain 1624	102	0.660	155
Strain 1605	255	0.850	300
Strain A-5	73	0.358	204
Strain 6718	119	0.430	277
Strain 6227	90.5	0.580	156
Strain 6472	56.2	0.350	160

Fractional Precipitation with Refractive Index Analysis

In order to determine the polydispersity of the most promising polysaccharides obtained from the screening procedure, a fairly accurate, though simple and rapid, method for fractional precipitation was developed. This method employs an Abbe refractometer with a sugar scale to measure the concentration of unprecipitated polysaccharide. Since a polysaccharide has an index of refraction similar to that of other sugars, one can consider the sugar scale to read per cent polysaccharide. The refractometer is connected to a constant temperature bath in the usual manner. The procedure followed in a fractionation was as follows: One hundred ml. of a 1 to 5 per cent solution of the polysaccharide was placed in a 600 ml. beaker. Methanol was added from a burette in varying increments while the solution was vigorously stirred with a magnetic stirrer. After each addition, one drop of the supernatant was removed and was analyzed for polysaccharide with the refractometer. The per cent (uncorrected) of polysaccharide in the supernatant was determined as the difference between the refractometer per cent reading for this solution and the reading for a solution containing the same proportion of water and methanol.

Since the original polysaccharide solution was effectively diluted by the added methanol, the apparent per cent of polysaccharide in the supernatant would show a decrease with each methanol addition. Therefore, a correction factor had to be

applied to determine the amount of polysaccharide actually precipitated. Also, addition of alcohol to water causes a shrinkage in the total volume; therefore, an additional factor had to be inserted to correct the dilution factor.

The maximum error in reading the refractometer is approximately 0.1 per cent. Since in the determination of concentration of polysaccharide in any given sample the per cent polysaccharide (uncorrected) is multiplied by the corresponding dilution factor, the error in the original per cent reading would also be increased by the same factor. This increase in maximum error with increasing methanol concentrations is shown in Figures 4, 5 and 6 by the height of the vertical lines on the refractive index curves.

It was found that the presence of very finely divided precipitate particles in the supernatant led to indistinct readings and, consequently, large errors in the apparent sugar per cent. This problem was overcome by sampling the supernatant with a piece of glass tubing of 2 mm. inside diameter capped on one end with a small piece of Whatman No. 44 filter paper. The supernatant drawn through the filter paper is completely free from these finely divided particles and is satisfactory for refractive index analysis.

In order to check the accuracy of the refractive index fractionation, successive fractional precipitations were carried out using an analytical method of established accuracy, namely the anthrone method. The solutions used for these fractionations were part of the same solutions made up for the refractive index analysis. In this procedure, twelve 16 x 150 mm. test tubes were set up containing polysaccharide solution to which had been added amounts of methanol to make the final concentration of alcohol in the tubes range from 0 to 80 per cent. The tubes were then stoppered tightly, mixed, and allowed to stand overnight in a cold room to insure complete precipitation. The following day, the tubes were centrifuged, and anthrone analyses were run on the precipitates.

Two bacterial polysaccharides (from cultures A-5 and 248) and one plant polysaccharide (an arabogalactan from western larch) were fractionated by the two procedures outlined above. The results of these fractionations are shown in Figures 4, 5 and 6.

As can be seen from the curves, the refractive index fractionation curve is almost identical with the more accurate anthrone curve in all cases.

Further Work on the Polysaccharide from Western Larch

As indicated in the last report (1), the polysaccharide obtained from western larch appeared to possess physical properties making it feasible for use as a plasma substitute. Therefore animal work was undertaken to determine the retention time of the polysaccharide in the blood stream.

The polysaccharide was partially purified by treatment with 15 per cent H_2O_2 followed by precipitation with acetone. Twenty ml. of a 20 per cent sterile aqueous solution were injected intravenously into a 3000 g. rabbit. Samples (0.1 ml.) of blood were removed at intervals, deproteinized with 10 ml. of 10 per cent trichloroacetic acid, centrifuged, and the supernatant measured for polysaccharide content by the following procedure, based on the method of Sorensen and Haugaard (6): Into matched 18 x 150 mm. test tubes were pipetted 1 ml. sample containing 5 to 30 µg. polysaccharide, 1 ml. of 1.56 per cent aqueous orcinol, and 5 ml. H_2SO_4 solution (350 ml. H_2SO_4 + 150 ml. H_2O). The tubes were heated at 100° C. for 15 minutes, cooled, and the per cent transmission read in an Evelyn colorimeter at 440 mµ. Appropriate blanks and standards were run with each set of samples.

Figure 4

Methanol Precipitation of the Polysaccharide from Culture A-5

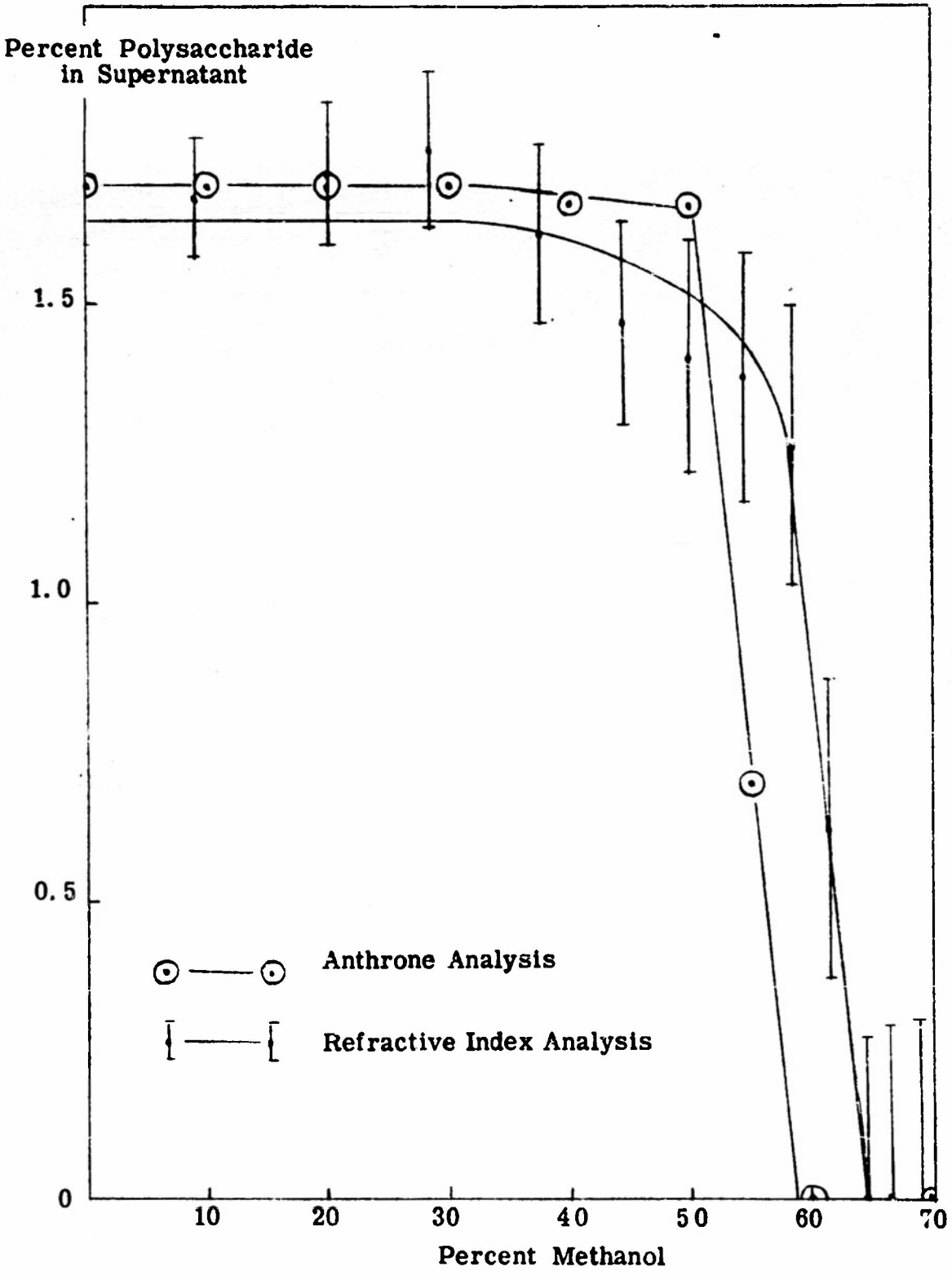


Figure 5

Methanol Precipitation of the Polysaccharide from Western Larch

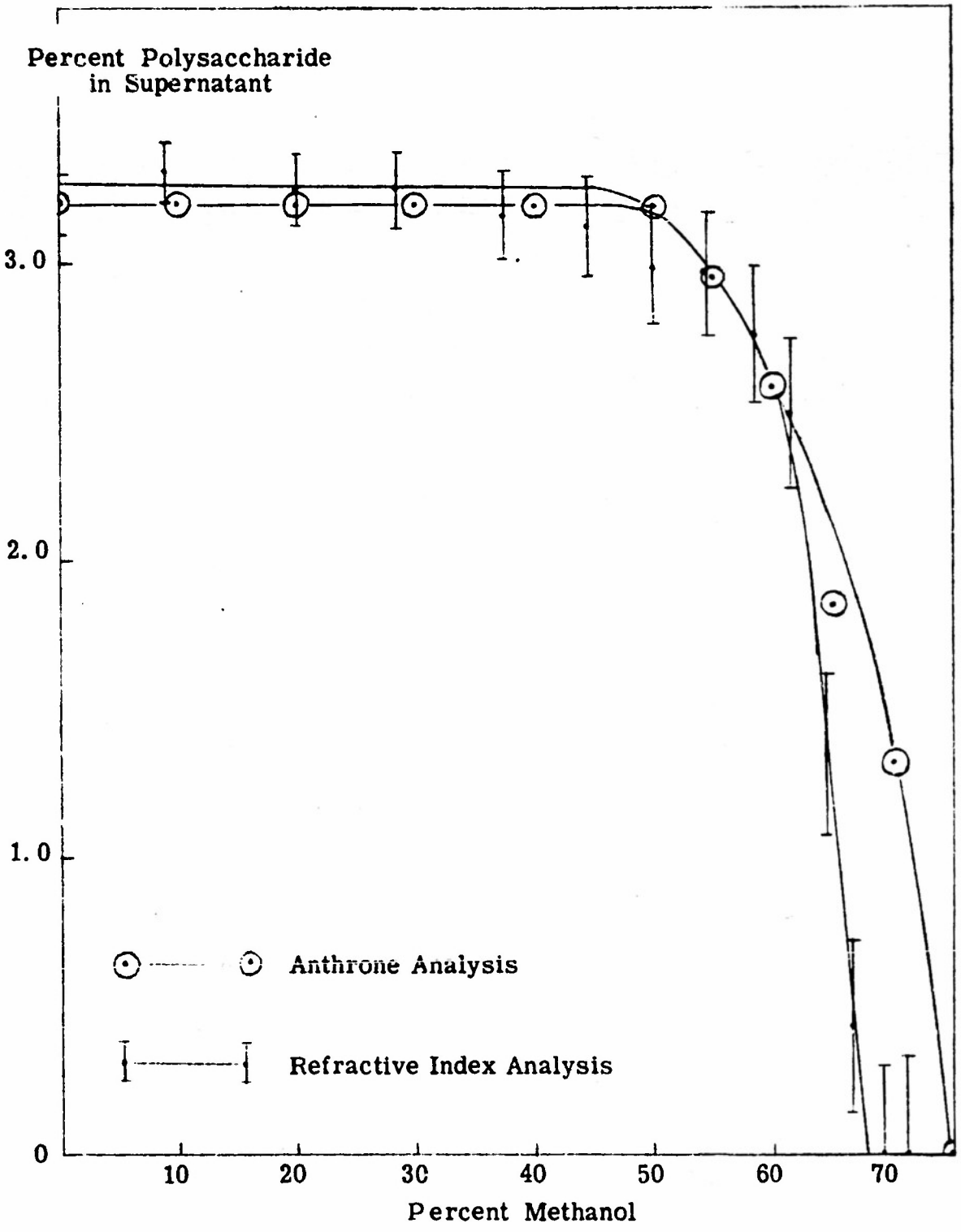
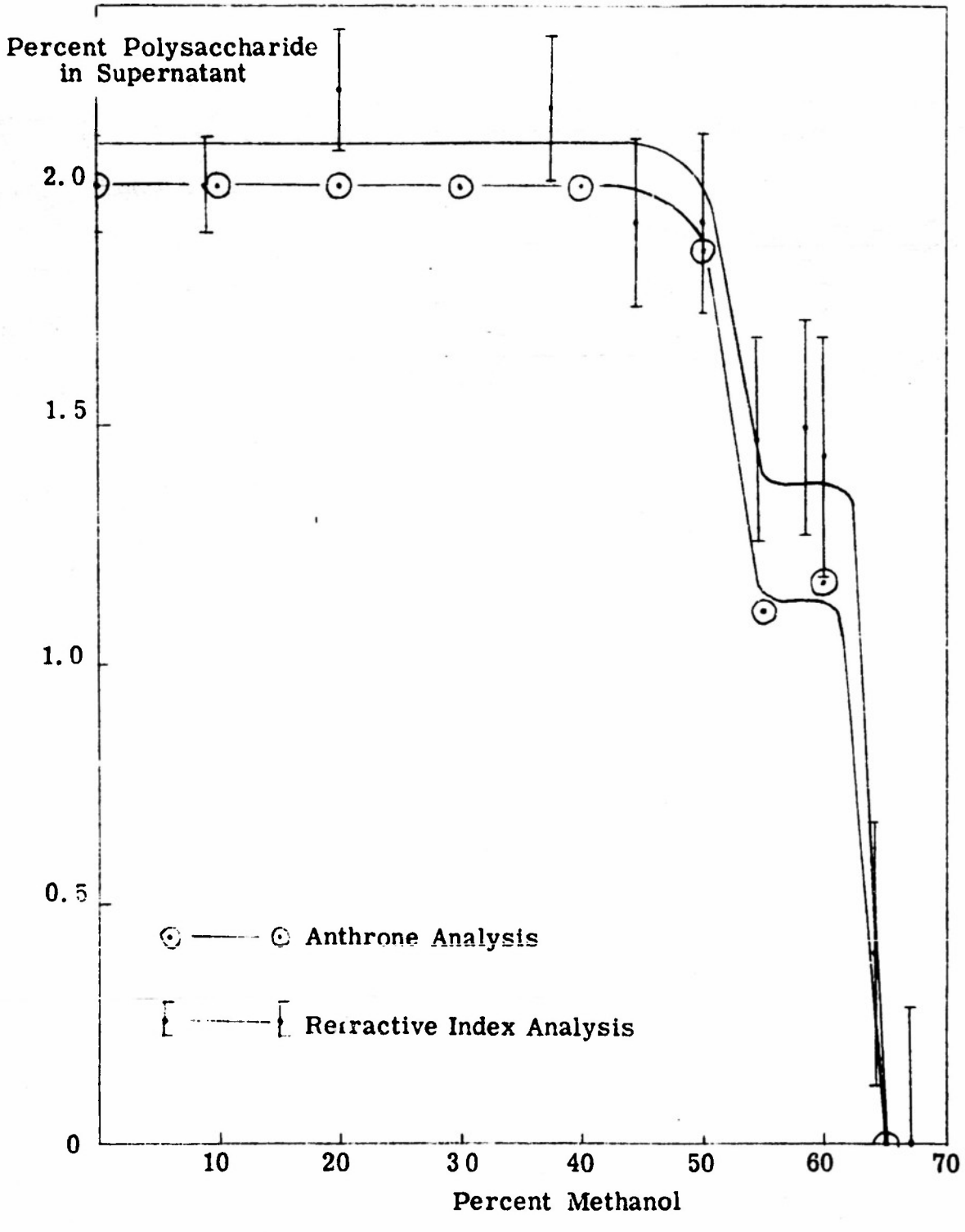


Figure 3

Methanol Precipitation of the Polysaccharide from Culture 248



It was found that only 10 per cent of the polysaccharide remained in the blood after two hours. As a control, 40 ml. of a 6 per cent solution of dextran 481 was injected into the same rabbit several weeks later. Seventeen per cent of the dextran still remained in the blood after 48 hours. Since it was apparent that the larch polysaccharide was lost from the blood far too rapidly to be of any value as a plasma substitute, work on it was stopped.

Animal Work

Preliminary animal work was done in an attempt to study blood recovery in a rabbit which had been hemorrhaged to an extent of 25 to 35 per cent of the total blood volume. As yet, no significant information has been obtained from determinations of blood pressure, blood volume, hematocrit, and plasma nitrogen.

Fermentation Studies on Strain 248

From the screening procedure a total of 7 cultures have been obtained which produced polysaccharides of desirable properties. One of these, strain 248, was selected for a study of the means whereby maximal yields of an optimal sized polymer might be obtained.

As indicated in the section contributed by the Department of Bacteriology, strain 248 is an aerobic spore-former and appears to be a variety of Bacillus subtilis. The polysaccharide produced by this microorganism possessed properties typical of levans, i.e. fructosidic polymers. In 0.4 N HCl the polysaccharide was completely hydrolyzed in less than 15 minutes at 100° C., and the hydrolysate, when chromatographed on Eaton-Dikemann 613 filter paper using a water, butanol, pyridine system (3-6-4 volume ratio), yielded only one spot, corresponding to that of fructose. In addition, the rate of formation of the color complex with the anthrone reagent was the same for 248 polysaccharide as for fructose. This suggests that the polymer consists of anhydrofructose units (7).

A. Methods. The amount of polysaccharide in the broth was determined by a selective elution method as follows: From 5 to 50 µl. of broth, after centrifugation of the cells, were applied to the center of a 3 cm. section of one-half inch wide Eaton-Dikemann 613 filter paper. It was convenient to make as many as 30 such applications on one strip of filter paper. After the filter paper had air-dried, it was immersed in about two liters of methanol, 90 per cent by volume, for 10 to 15 minutes. The strip was then subjected to one rinse (5 minutes) in a second bottle containing the same concentration of methanol. When the strip had air-dried, it was cut into its numbered sections, which were then placed in similarly numbered test tubes. Four ml. of approximately 0.1 N HCl were added to the tubes containing blanks and samples. Standards were included by adding 2 ml. of a standard fructose solution, containing approximately 40 gamma fructose per ml., and 2 ml. of approximately 0.2 N HCl to tubes containing blank sections of the original strip of filter paper. In this way, any extraneous carbohydrate picked up by the elution procedure would be discounted, since blanks and standards should contain the same amount of background eluate. Elution with acid was allowed to proceed for 10 to 15 minutes. Two ml. of each eluate were then transferred to standard tubes for anthrone assay. These conditions are satisfactory for assaying a broth which contains from 1 to 25 mg. of polysaccharide per ml. Suitable alterations in the procedure can be made if higher or lower concentrations of polysaccharide are present.

Under the conditions of this assay essentially all of the residual sucrose in the broth, along with any reducing sugars and low molecular weight polysaccharides, are removed by the elution with 90 per cent methanol. The elution with 0.1 N HCl removes the higher molecular weight polysaccharides. The results obtained in this way are 0 to 10 per cent higher than the values obtained by a method involving

dialysis through cellophane for 48 hours, followed by an anthrone assay of the non-dialyzable material. The advantage of the selective elution method lies in the speed with which the amount of polysaccharide in a broth can be determined.

An extension of this selective elution procedure, employing methanol concentrations of 0 to 90 per cent methanol, has been of value as a fractionation technique. The procedure in this case is much the same as described above, with the exception that only 60 to 70 ml. of each methanol concentration were used for the alcohol elution and rinse. This phase of the procedure is conveniently carried out in 200 x 25 mm. test tubes. Four polysaccharide samples, run in duplicate, can be fractionated simultaneously in this manner. The time of methanol elution is somewhat more critical with methanol concentrations lower than 90 per cent; probably because the larger molecules, which would tend to go into solution at the lower methanol concentrations, dissolve at a slower rate. Reproducible results are obtained, however, when 15 minutes are allowed for elution of low molecular weight material. Neither temperature (26 to 37° C.) nor volume of eluent (60 to 500 ml.) are critical in the methanol elution step. The fraction of polysaccharide not eluted from the filter paper by water is termed "high molecular weight" polysaccharide.

The amount of reducing sugar in the cell-free broth was analyzed by the method of Shaffer and Somogyi (8).

The nitrogen content of polysaccharide preparations was determined by a convenient micro-Kjeldahl method (5).

The determination of the intrinsic viscosity of a polysaccharide, while still in the cell-free broth, was made by a procedure described elsewhere (1).

Calculation of number average molecular weight from osmotic pressure data was accomplished as related in last year's report (1).

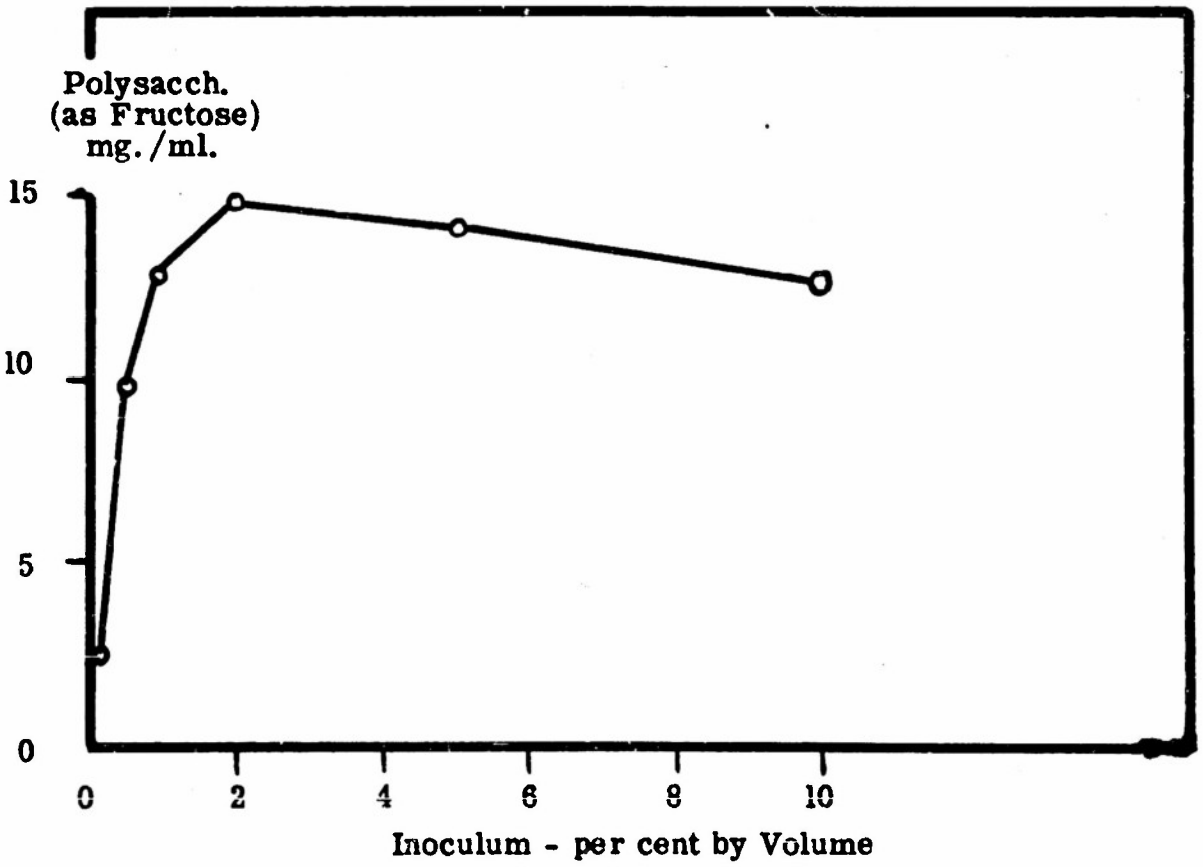
Nitrogenous impurities in a polysaccharide preparation were largely removed by a deproteinization procedure. A 5 per cent solution of the polysaccharide was made in pH 5 M/10 acetate buffer. This solution was heated in a boiling water bath for 20 minutes. The light tan precipitate which formed was centrifuged off. From hydrolysis rate studies on the polysaccharide, it has been calculated that no more than 0.1 per cent hydrolysis could have occurred during these conditions of deproteinization.

Weight average molecular weight data on polysaccharide preparations were obtained by light scattering measurements with a Bryce-Phoenix photometer. A monochromatic light source of λ = 436 mμ was employed. It was assumed that the particle in solution were small and isotropic. Calculations of molecular weights were made by using the equations developed by Debye (9) for small particles in concentrated solutions.

B. Effect of inoculum on polysaccharide production. The amount of inoculum employed was found to be very important in determining the ultimate yield of polysaccharide. This was demonstrated as follows: Ten ml. of basal synthetic medium (1) in a 25 x 200 mm. tube were inoculated by loop from an agar slant of strain 248 growing on the same medium. The tube was incubated at 30° C. on a rotary shaker for 24 hours. Another tube containing fresh medium was then inoculated with 0.2 ml. of the 24 hour culture. After two successive transfers of 24 hour old cultures, varying amounts of the culture were inoculated into 100 ml. of basal synthetic medium in 500 ml. Erlenmeyer flasks. The flasks were incubated at 30° C. on a rotary shaker. After 24 hours the broths were assayed for polysaccharide by the selective elution method. The results are shown in Figure 7. It may be noted that polysaccharide production was markedly stimulated as the inoculum amount increased

Figure 7

The Effect of Inoculum Size on Polysaccharide Production



to 2 per cent. A larger inoculum decreased the yield of polysaccharide.

It might be assumed that if the flasks with less inoculum were incubated longer, polysaccharide would be formed in an amount equal to that obtained when a 2 per cent inoculum was employed. This has proved not to be the case. Figure 8 indicates that while polysaccharide continued to be synthesized for a longer period of time with a 0.1 per cent inoculum, the total amount produced never attained the value observed when more inoculum was used.

It was thought that this inoculum effect might be attributable to either a polysaccharide primer or levancucrase transmitted with the inoculum. This idea was tested by using a 0.1 per cent inoculum and adding to it enough centrifuged broth to make the total volume equal to 2 per cent. In one instance the broth was heated for 5 minutes at 100° C. to inactivate any enzymes present. As indicated in Figure 8 some further production of polysaccharide was obtained, but not as much as when the larger number of cells was used for inoculum. It may be concluded that this inoculum effect is caused by some property of the cells themselves.

C. Effect of incubation temperature on polysaccharide production. A variation of 5° C. in the incubation temperature was found to alter the course of the fermentation appreciably. As shown in Table 4, a culture of 248, when incubated in basal

Table 4

Incubation Temperature vs. Production of Polysaccharide and Reducing Sugar

Time Hrs.	25° C. Concentration Red. sugar %	25° C. Concentration Polys. %	30° C. Concentration Red. sugar %	30° C. Concentration Polys. %
24	2.7	0.96	0.58	0.68
48	1.58	0.91	1.24	0.36
72	1.06	0.62	0.8	0.03

synthetic medium at 25° C., produced more polysaccharide than it did at 30° C. Moreover, at the lower temperature, the polysaccharide remained in the broth for a longer period of time. Some insight as to the utilization of the sucrose in the original medium is also indicated in Table 4. After 24 hours of growth at 25° C., more reducing sugar than polysaccharide was present, and both of these figures were higher than the corresponding values at 30° C. From 24 to 48 hours, the 25° cells metabolized the reducing sugar which had accumulated during the first 24 hours. The 30° cells, however, continued to metabolize the sucrose, and also began to hydrolyze the accumulated polysaccharide. In the final 24 hour period of the fermentation the reducing sugar and polysaccharide were metabolized at both temperatures, though both sources of carbohydrate were more rapidly depleted at 30° C.

Table 5 contains data on the percentage of "high molecular weight" polysaccharide present in the broth at various times during the fermentations at 25° and 30° C. The term "high molecular weight polysaccharide" represents that amount of polysaccharide which is not eluted by water from Eaton-Dikemann No. 613 filter paper. It may be seen that this fraction increased in amount as the fermentation proceeded beyond 24 hours. Although this phenomenon occurred at both 25° and 30° C., it is apparent that the effect was more pronounced at the higher temperature. Since this increase in the high molecular weight fraction was attended by a decrease in

Figure 8

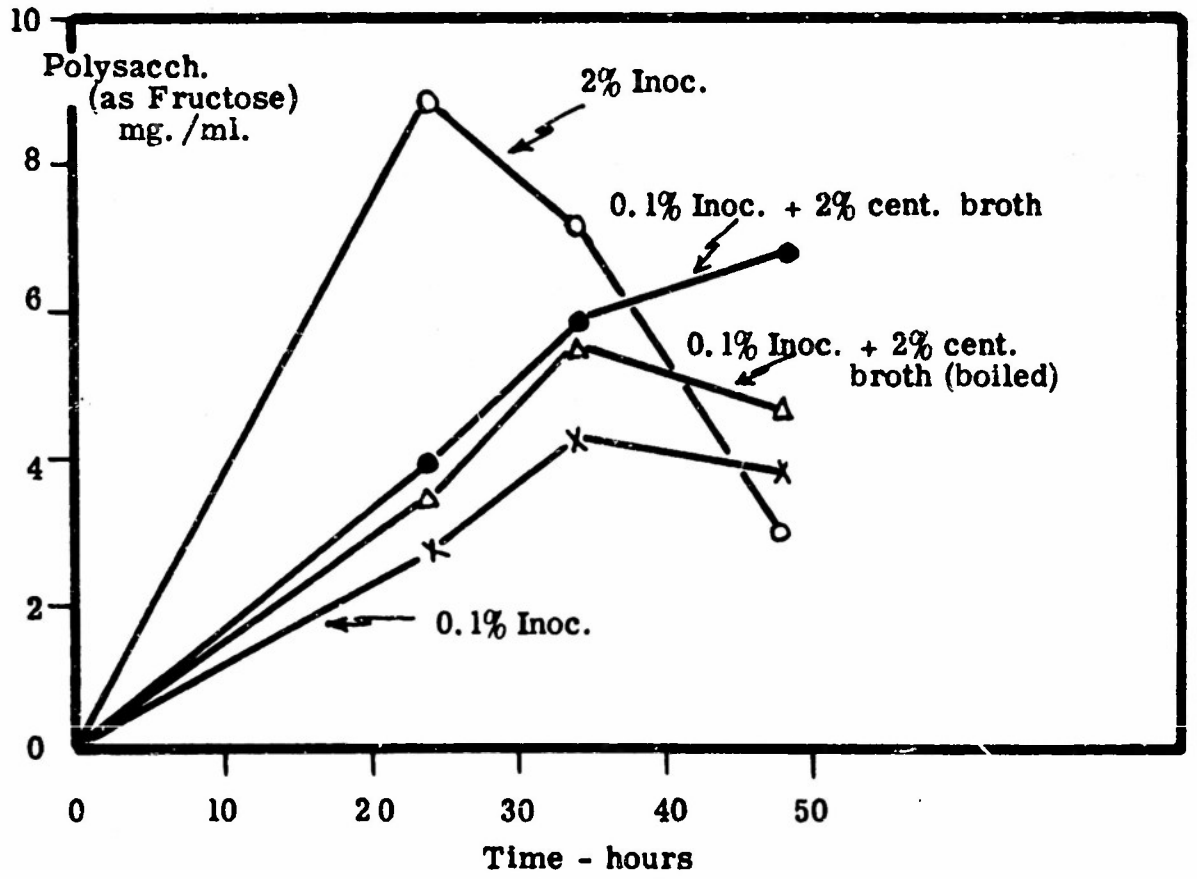

10
8
6
4
2
0
Polysacch.
(as Fructose)
mg./ml.
2% Inoc.
0.1% Inoc. + 2% cent. broth
0.1% Inoc. + 2% cent.
broth (boiled)
0.1% Inoc.
0
10
20
30
40
50
Time - hours

Table 5

Incubation Time vs. Amount High Molecular Weight Polysaccharide at 25° C. and 30° C.

Time Hrs.	25° C. High mol. wt. polys. % of total	25° C. Intrinsic viscosity	30° C. High mol. wt. polys. % of total	30° C. Intrinsic viscosity
24	24.6	0.32	25.6	0.33
48	34.6	0.42	48.7	0.52
72	46.9	0.64		

the total polysaccharide present (as shown in Table 4), it may be concluded that the apparent increase of high molecular weight material is only a reflection of the more rapid disappearance of low molecular weight material.

Crude measurements of intrinsic viscosity of the polysaccharide in the broth signify the same trend (see Table 5), i.e., a larger proportion of high molecular weight material as the fermentation proceeded beyond 24 hours.

D. Growth experiments with various media. The medium employed in the screening procedure is described elsewhere (1). Since it was thought that strain 248 might not require such a complete medium, tests were performed to determine what constituents of the medium might be omitted.

Uniform amounts of inoculum from a culture of 248 growing on the basal synthetic medium were added to each of a number of 18 x 150 mm. test tubes containing 5 ml. of medium of varying composition. Growth was followed turbidimetrically in an Evelyn colorimeter with a 660 mμ filter. The results of this experiment are shown in Table 6.

Table 6

Growth Experiments with Strain 248

Constituents in medium*	P = Present										
Sucrose	P	P	P	P	P	P	P	P	P	P	P
Inorganic salts	P	P	P	P	P	P	P	P	P	P	P
Purines and pyrimidines	-	P	P	P	-	-	P	-	P	P	-
Vitamin sol'n. "A"	-	-	P	-	P	P	P	-	P	P	-
Vitamin sol'n. "B"	-	-	-	P	-	P	P	-	-	P	-
Casamino acids	P	P	P	P	P	P	P	-	-	-	-
Ammonium citrate	-	-	-	-	-	-	-	P	P	P	-
Yeast extract (0.5%) + tryptone (1%)	-	-	-	-	-	-	-	-	-	-	P
660 mμ Galvanometer Reading after 15 Hours Growth (1/10 dil.) % Transmission	48	46	51	50	44	56	38	70	62	60	52

* The amounts of various constituents are those which are listed elsewhere for basal synthetic medium (1). Vitamin sol'n. "A" contained thiamin-HCl and riboflavin. Vitamin sol'n. "B" contained all the other vitamins listed in the basal synthetic

medium. Ammonium citrate was used to replace the Casamino acids on an equivalent nitrogen basis.

It may be seen that the culture of 248 grew well even though purines, pyrimidines, vitamins and other growth factors were omitted from the medium. When dibasic ammonium citrate was used as the sole nitrogen source (replacing Casamino acids on an equivalent nitrogen basis), good growth was observed, though the growth rate was somewhat slower. A yeast extract, tryptone medium, used for purposes of comparison, showed no better growth than the simple Casamino acids medium (medium 115-1).

In other experiments, ammonium acetate was used as the sole nitrogen source. Since the growth rate was markedly reduced it was concluded that the acetate anion was in some way acting as an inhibitor.

Polysaccharide production by 248 grown on 115-1 medium as compared to basal synthetic medium is shown in Table 7.

Table 7

Polysaccharide Production by Strain 248 at 25° C.

Time Hrs.	Basal syn. med. polys. as fruct. mg./ml.	115-1 Med. polys. as fruct. mg./ml.
24	6.7	12.0
33	10.5	14.3
48	9.9	13.9

Equivalent amounts of 24 hour old cultures, grown on the respective media, were introduced into 100 ml. of each medium in 500 ml. Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 25° C., and tested periodically for pH and polysaccharide. From Table 7 it is evident that as much or more polysaccharide was elaborated in the simpler medium (115-1).

It was found that polysaccharide synthesis was unimpeded by the substitution of ammonium sulfate for the Casamino acids in the medium. Because the pH might be expected to drop to a greater extent when ammonium sulfate is used, measures were taken to check this tendency. Table 8 lists the composition of the media employed. The amount of polysaccharide produced in each of these media is shown in Table 9.

E. Data on molecular weights. Strain 248 was grown on 115-1 medium (using 10 per cent sucrose rather than 5 per cent sucrose) under conditions described earlier as being most effective in producing high yields of polysaccharide; i.e., with a 2 per cent inoculum of a 24 hour old actively growing culture, and incubating at 25° C. on a rotary shaker. After removal of cells by centrifugation, the supernatant was heated for 3 minutes at 100° C. to prevent further enzymatic action. The polysaccharide was then precipitated by adding methanol to 85 per cent by volume. The precipitate was redissolved in water (to give approximately a 4 per cent solution) and reprecipitated twice more in the same fashion. This procedure sufficed to remove essentially all the residual sucrose and reducing sugars. The final precipitate was dried overnight in a vacuum oven at 65° C. Ash and nitrogen analyses, as well as molecular weight data, are shown in Table 10. The nitrogen content was reduced by a deproteinization procedure described in Methods.

Table 8

Composition of Media

Composition	6153-1	6153-2	6153-3	6153-4	6153-5	115-1
Sucrose (g./100 ml.)	5.0	5.0	5.0	5.0	5.0	5.0
$(NH_4)_2SO_4$ "	0.47	0.47	-	-	-	-
Casamino acids "	-	-	1.0	1.0	-	1.0
Salts A* (ml.)	-	-	20.0	20.0	-	20.0
Salts B** (ml.)	0.5	0.5	0.5	0.5	0.5	0.5
K_2HPO_4 (g./100 ml.)	2.0	0.05	1.9	-	0.05	-
KH_2PO_4 (g./100 ml.)	-	0.05	-	-	0.05	-
$CaCO_3$*** (g./100 ml.)	-	0.5	-	0.5	-	-
Yeast extract (g./100 ml.)	-	-	-	-	0.5	-
Tryptone (g./100 ml.)	-	-	-	-	1.0	-

The pH of all media was adjusted to 6.8 before autoclaving.

* Salts A contains tryptophan, cysteine and phosphate in amounts equal to those in basal synthetic medium (1).

** Salts B contains all other inorganic salts as listed for basal synthetic medium.

*** Sterilized separately as a 25 per cent slurry.

Table 9

Polysaccharide Production at 25° C. on Synthetic Media

Time Hrs.	Media 6153-1	6153-2	6153-3	6153-4	6153-5	115-1
	mg./ml. as fructose					
24	7.8	6.5	5.3	7.2	7.4	7.8
32	8.0	9.2	7.7	7.3	7.5	8.0
48	6.1	7.6	8.6	7.6	6.4	7.5
			--- pH ---			
24	5.8	5.0	6.4	5.6	5.7	5.5
32	5.6	4.8	6.6	5.9	5.6	5.5
48	5.7	4.8	6.4	6.0	5.3	3.7

Table 10

Molecular Weight Data on Strain 248 Polysaccharide

Sample	Ash %	N %	Wt. ave. mol. wt.	No. Ave. mol. wt.
248 Polys.*	0.86	0.26	2.2×10^6	31,000
Same - deprot.	-	0.08	0.75×10^6	-

* Harvested from 10% sucrose, Casamino acids medium.

It may be noted in Table 10 that the deproteinization treatment effected both a decrease in the nitrogen content and a reduction in the weight average molecular weight. It is believed that the reduction in weight average molecular weight is due to the removal of partially insolubilized protein, which, when present in suspension, would cause a high degree of light scattering.

The deproteinized polysaccharide was subjected to fractionation with methanol, and the weight average molecular weights of these fractions were determined by light scattering measurements. From these data, shown in Table II, it may be seen that one fraction, which constituted 25 per cent of the total polysaccharide, represented material which may be satisfactory as a plasma substitute.

Table 11

Molecular Weight Data on Methanol Fractions of Strain 248 Polysaccharide*

Fraction No.	MeOhanol Vol. %	Pptd. polys. % of total	Wt. ave. mol. wt.	Polys.** % of total
1	55.6	13.5	5,500,000	62.9
2	64.8	2.3	—	—
3	70.0	25.2	45,500	4.9
4	74.2	15.3	—	—
5	78.5	20.9	15,400	2.0
6	90.0	22.6	—	—

* Grown on 10% sucrose, Casamino acids medium 115-1.
** Polys. not eluted from filter paper by water.

Another fraction, 13 to 15 per cent of the total, was of high molecular weight, and the remainder was probably of too low a molecular weight to be useful as a plasma substitute. In this same table it may be seen that a good correlation exists between molecular size and the percentage of the total polysaccharide which is not eluted from filter paper by water.

It should be pointed out that the degree of homogeneity of the 45,500 molecular weight fraction has not yet been established.

Summary

(1) In last year's annual report a screening procedure was described for the isolation of microorganisms which produce polysaccharides that might be useful as plasma substitutes without extensive preparation. This screening procedure has been improved by the development of a large-pore membrane, which more closely approximates, in a 96 hour dialysis, the excretion of clinical dextrans from the kidneys in 24 hours.

(2) Of the 1000 cultures studied, 7 passed the screening procedure and have been retained for further study.

(3) A rapid method for fractional precipitation is described. This method employs an Abbé refractometer to measure the concentration of unprecipitated polysaccharide.

(4) Strain 248, which passed the screening procedure, is a variety of *Bacillus subtilis*. The polysaccharide synthesized by this microorganism from sucrose is a levan.

(5) A rapid assay method for determining polysaccharide in crude broth is described. An extension of this method provides data on the number of fractions in the crude polysaccharide.

(6) With strain 248, variations in the amount of inoculum and incubation temperature have proved to be very important in determining the final yield of polysaccharide, its stability in the medium, and the amount of polysaccharide which is of high molecular weight.

(7) The molecular weight of several fractions obtained by methanol precipitation of 248 polysaccharide has been measured by light scattering. One fraction, which constituted 25 per cent of the total polysaccharide, possessed a weight average molecular weight of 45,500. The degree of homogeneity of this fraction has, however, not yet been established.

(8) The polysaccharide obtained from the western larch tree has been found to be excreted far too rapidly to be of any value as a plasma substitute.

References

1. "Microbial Polysaccharides as Plasma Substitutes", ONR Contract No. N7onr-28508, Annual Report. (June 1, 1951 to June 30, 1952).

2. Pulaski, E. J., Quart. Rev. Med., August, 1952, p. 44.

3. Stavely, H. E., Private Communication.

4. Houlihan, R. B., Private Communication.

5. Johnson, M. J., J. Biol. Chem., 137, 575 (1941).

6. Sorensen, M., and Haugaard, G., Compt. rend. trav. lab. Carlsberg, 19, no. 12 (1933).

7. Koehler, L. H., Anal. Chem., 24, 1576 (1952).

8. Shaffer, P. A., and Somogyi, M., J. Biol. Chem., 100, 695 (1933).

9. Debye, P., J. Phys. Coll. Chem., 51, 18 (1947).